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The Effects of a Reservoir on Genetic Isolation in Two Species of Darters

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THE EFFECTS OF A RESERVOIR ON GENETIC ISOLATION IN TWO SPECIES OF DARTERS

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THE EFFECTS OF A RESERVOIR ON GENETIC ISOLATION IN TWO SPECIES OF DARTERS

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The addition of dams into a riverine system causes a wide range of changes (i.e., sedimentation, erosion, thermal) to the river as well as to the fish assemblages of that river. Although there have been many studies documenting the changes that occur to the fish assemblages in the impounded river, there have been fewer studies examining the effects of a reservoir on the fish inhabiting the tributaries upstream of the impoundment. One possible impact of a reservoir could be to act as a barrier to fish migration between streams.

To determine if reservoirs restrict migration, the genetic diversity of two species of darter, the rainbow darter *Etheostoma caeruleum* Storer and the Highland Rim darter *Etheostoma kantuckeense* Ceas and Page, was determined from populations inhabiting the Barren River Lake drainage basin. Between ten and twenty-six individuals of each species were collected from each of 6 sites. Three streams were directly connected to Barren River Lake and three streams were directly connected to Barren River upstream of the reservoir. Allelic variation at 3 microsatellite loci was analyzed to determine the degree to which each population is isolated. If the reservoir is restricting gene flow

between populations, the populations in streams adjacent to Barren River Lake would be predicted to have lower allele diversity and heterozygosity than those adjacent to the Barren River.

Consistently high levels of allelic diversity (total number of alleles, N), observed heterozygosity (H_o), and effective number of alleles (A_e) across both reservoir and river study sites led to the rejection of the hypothesis that the reservoir is acting as a genetic barrier to darters. *M*-ratios differed between species, with *Etheostoma caeruleum* exhibiting consistently higher *M*-ratios than *Etheostoma kantuckeense*. The low *M* seen in *E. kantuckeense* could be due to small sample sizes (largest sample for this species showed the highest *M*), and could also be due to small natural populations. With the exception of Salt Lick Creek, high allelic diversity was observed at most sites for *E. kantuckeense*. A low *M,* coupled with high allelic diversity in most *E. kantuckeense* populations, may indicate that all of the study populations are recovering from a bottleneck event.

These results indicate *Etheostoma kantuckeense* is sensitive to changes in the environment. When conservation agencies assess fish populations in South Central Kentucky, it is advantageous to know which species are currently at risk, which species are sensitive to environmental changes, and which species or populations are recovering from events that were detrimental to their genetic diversity.

Introduction

Considerable research effort has been dedicated to observing the effects of dam construction and reservoir formation on fish communities within the reservoir basin and the surrounding environment (Baxter 1977; Timmons et al. 1978; Martinez et al. 1994; Bonner and Wilde 2000; Lienesch et al. 2000; Phillips and Johnston 2004; Falke and Gido 2006; Matthews and Marsh-Matthews 2007). Many of these studies show that once abundant fish populations have drastically decreased or disappeared as a result of changes in river volume and channel morphology as well as loss of refugia and spawning habitat associated with the construction of reservoirs and dams (Lienesch et al. 2000; Bonner and Wilde 2000; Falke and Gido 2006; Matthews and Marsh-Matthews 2007). The intent of this study is to determine how the construction of reservoirs may be affecting populations of two fish species, the rainbow darter (*Etheostoma caeruleum* Storer) which is widely distributed throughout Eastern North America and the Highland Rim darter (*Etheostoma kantuckeense* Ceas and Page) which is endemic to the Barren River Drainage in South Central Kentucky.

Dams and reservoirs are used throughout the world to control flooding, generate electricity, and the store drinking water (Dynesius and Nilsson, 1994). Reservoirs are a major cause of water evaporation throughout the world. Thirty-two percent of the runoff from the Colorado River system is lost through evaporation from reservoirs (Dynesius and Nilsson, 1994), which causes changes to many aspects of the environment and fish communities that inhabit the impounded rivers.

When a dam is constructed and a reservoir formed, the most obvious change seen is the change in water flow in the impounded river (Bonner and Wilde 2000). The once lotic environment is abruptly changed into a lentic environment, causing many changes to the environment. When a river that carries a large load of sediment reaches a reservoir, the sediment is deposited on bottom of the reservoir and causes many problems for the benthic fish as well as the fish that would lay their eggs under rocks at the bottom of a river. If the gradient of the impounded river is not steep enough, the sediment may be dropped before reaching the reservoir, causing the river flow to change upstream of the reservoir.

Dams and reservoirs not only affect the immediate area, but they also bring about changes to the river downstream. When water is released from a reservoir it is usually free of sediment, including sand and silt, but also particulate organic matter. The lack of organic matter from upstream results in the depletion of a major source of food for many aquatic organisms including macroinvertebrates, which are a major food source for many species of fish, including darters (Baxter 1977).

Other changes that occur when a reservoir forms affect the thermal makeup and oxygen content of the water. Because the flow in rivers and streams is almost always turbulent, there is a constant mixing of oxygen into the system. Turbulent flow also distributes heat absorbed from solar radiation striking the surface. Once water enters a reservoir where there is no turbulent flow, thermal stratification occurs. Heat from solar radiation generates the epilimnion, a layer of warm, low-density water at the surface. Because of the difference in density, the epilimnion rarely mixes with the hypolimnion, the cool, high-density water at the bottom of the reservoir. The lack of mixing between

the epilimnion and hypolimnion will result in differences in dissolved oxygen concentration. Because there is little mixing between the epilimnion and hypolimnion, oxygen absorbed from the atmosphere at the surface of the lake is not carried to the deeper layers of the reservoir. Respiration by microbes and decomposition of organic matter at the bottom of the reservoir also lead to decreased dissolved oxygen levels in the hypolimnion. Decomposition of organic matter by microbial life contributes to anoxia in the deepest waters of a reservoir, and also releases sulfide, ferrous and manganous ions from sediment (Baxter 1977). These complex processes may change the physico-chemical parameters of the hypolimnion, such as oxygen content and temperature, making it inhospitable for native fish.

When a river is abruptly transformed into a reservoir, there are changes that occur which could be harmful to the fitness of individuals of many fish species and often leads to dramatically decreased populations or local extirpations of native fish species (Timmons et al. 1978; Martinez et al. 1994; Bonner and Wilde 2000; Phillips and Johnston 2004). One change that can have a major effect on a fish population is the loss of spawning and nursery habitat (Baxter 1977). Many species of riverine fish use the riparian vegetation for protection of their eggs and larvae. After a reservoir is formed, large fluctuations in water level due to seasonal outflow causes shorelines to erode. When this happens, the gentle sloping bank of a river becomes a steep embankment on which riparian vegetation is unable to establish itself. Without riparian vegetation to use as protection for young, some fish species have difficulty maintaining a healthy population size.

When there is ample vegetation present on the shoreline of a reservoir, there are sometimes still problems that plague the near shore fish populations that utilize that vegetation. Because water may be released from a reservoir during the spawning season of many fish, any fish eggs or larvae that inhabit riparian vegetation at the time of release will die because the once inundated riparian vegetation is now exposed (Baxter 1977). The decrease in flooding events caused by reservoirs is also a problem for many primarily riverine fish. Species such as the plains minnow (*Hybognathus placitus* Girard), the Arkansas River shiner (*Notropis girardi* Hubbs and Ortenburger) and the flathead chub (*Platygobio gracilis* Richardson) rely on flood events to increase stream currents to keep their semi-buoyant and non-adhesive eggs viable and suspended in the water column. The currents associated with flooding events disperse newly hatched individuals to colonize downstream environments (Bonner and Wilde 2000). When semi-buoyant eggs are flushed into a reservoir, the eggs sink to the bottom and become covered by silt and asphyxiated. Fish parasites are also a problem in reservoirs. Zooplankton serves as an intermediate host for many fish parasites. Because zooplankton population levels rise dramatically in a reservoir, there becomes more of an opportunity for parasite populations to thrive (Baxter 1977).

When determining the impact a dam and reservoir has on a fish community in the impounded river, all of these variables are important to consider. Rare, endemic, and environmentally sensitive fishes are also important to examine, because these fish may not have a large influence on species composition indices because these fish are usually present in small numbers. This would mean that they would not have a large enough impact on species composition to be noticed until they have already disappeared (Phillips

and Johnston 2004). It is important not only to look at common species, but to also look for those species which might serve as indicator species for the health of the river system.

Dam construction not only affects the mainstem of the river, but also smaller tributaries to that river. A significant portion of tributary-type habitat may be lost when the river begins to transform into a reservoir, which may cause many fish to move upstream or become extirpated. Studies show that the fish assemblages shift when they inhabit a stream that is directly connected to a reservoir (Lienesch et al. 2000; Falke and Gido 2006; Matthews and Marsh-Matthews 2007) However, it has been suggested that those changes are highly localized at the confluence with the reservoir (Falke and Gido 2006), allowing reservoir species to invade the lower reaches of the stream. Loss of habitat in lower reaches on an inundated stream as well as isolation from remaining appropriate habitat has caused the extirpation of some populations of stream fish such as the Plains killifish (*Fundulus zebrinus* Jordan and Gilbert), the Topeka shiner (*Notropis topeka* Gilbert), the Carmine shiner (*Notropis percobromus* Cope), the brook silverside (*Labidesthes sicculus* Cope), and the ghost shiner (*Notropis buchanani* Meek) (Lienesch et al. 2000; Falke and Gido 2006), and dramatic declines in populations of other species such as the sand shiner (*Notropis stramineus* Cope), the Western silvery minnow (*Hybognathus argyritis* Girard), and the peppered chub (*Macrohybopsis tetranema* Girard) (Falke and Gido 2006).

Populations of reduced genetic diversity have difficulty surviving in the face of large or small-scale environmental changes. If populations are isolated by a change in habitat, then not only are they constrained to the immediate surrounding area, but also susceptible to localized environmental change (Saillant et al. 2004). Drought is one such

environmental change. When a drought occurs under natural stream conditions, many of the fish inhabiting that stream, including minnows and darters, find refuge downstream until conditions improve. However, when the stream is directly connected to a reservoir, the fish no longer have appropriate habitat for refuge. Instead of taking refuge in the reservoir where large piscivorous fish thrive, the smaller minnows and darters will shelter in isolated pools remaining within the streambed. If the drought continues those isolated pools dry up leaving the fish little chance of survival (Matthews and Marsh-Matthews 2007).

The physical barrier of a dam causes many problems for migratory fish, which may also suffer a loss of genetic diversity. Dams prevent upstream migration of many fish species, including those that rely on upstream sites for reproduction. Although fish are not able to swim upstream through a dam, many fish become caught in the outcurrent as water goes though the dam, flushing them downstream. This creates a one-way movement of genes within a river system, resulting in higher genetic diversity downstream and reduced genetic diversity upstream of the dam (Yamamoto et al. 2004). Populations of bull trout (*Salvelinus confluentus*) living upstream and downstream of Cabinet Gorge Dam on the Clark Fork River system are genetically distinct from each other, with the exception of one population in a tributary directly below the dam (Neraas and Spruell 2001). This could be due to reservoir spillover that occurs when flooding events occur (Neraas and Spruell 2001). Dams also impact populations of grayling (*Thymallus thymallus*) living within the Skjern River system in Denmark. Populations of grayling within the system are similar to each other with the exception of populations living in streams upstream of fish farms, which have installed weirs to help with water

retention. These weirs are acting negatively on the genetic diversity of grayling populations, isolating them from other populations downstream of the weir (Meldgaard et al. 2003).

The theory of island biogeography states that islands, or isolated populations, that inhabit a small area are more susceptible to catastrophic events (Macarthur and Wilson 1963). Catastrophic events diminish genetic diversity and decrease probability of survival. When a small population becomes genetically isolated, genetic drift and inbreeding cause the allele frequencies to change. Changes in allele frequency lead to the population losing rare alleles and becoming more homozygous. Although this loss in genetic diversity is not instantaneous, it is important to be able to identify genetic isolation as it is occurring. If genetic isolation can be identified early, ways to conserve the population can be put to action.

The fish species chosen for this study are *Etheostoma caeruleum* Storer and *Etheostoma kantuckeense* Ceas and Page. *Etheostoma kantuckeense* was described in 1997 from the *Etheostoma spectabile* species complex (Ceas and Page, 1997), but in 1968 Dan Distler first noted that the Barren River population of *E. spectabile* was distinctly marked (Distler, 1968). At the time of description *E. kantuckeense* was the third species of fish known to be endemic to the Barren River system (along with the blackfin sucker *Thoburnia atripinnis* Bailey and the splendid darter *Etheostoma barrenense* Burr and Page). *Etheostoma kantuckeense* is a member of the *E. spectabile* complex and is syntopic and easily confused with *Etheostoma caeruluem* (Ceas and Page, 1997). Distinctions can be made between the two species by comparing caudal ray counts, anal fin coloration, and lateral bar counts (Etnier and Starnes, 1993). Populations

of *E. caeruleum* in the Green River watershed, which contains the Barren River watershed, are thought to have relatively high levels of diversity due to the area having not been glaciated during the Pleistoscene (Ray et al., 2006). Because both species of darter are considered indicators of stream health and sensitive to their environment, the idea is that any change in their environment (including habitat fragmentation) would be evident relatively quickly in these species.

Genetic isolation would be caused by conditions in the reservoir that are not compatible with the life history of *E. caeruleum* and *E. kantuckeense*. First, darters usually inhabit fast flowing, riffle habitat that is not available in a reservoir. Second, darters feed almost entirely on benthic aquatic invertebrates which are unavailable in a reservoir. Also, the high rate of sedimentation in a reservoir may adversely affect the benthic eggs resulting in low reproductive success (Etnier and Starnes, 1993). The predators in a reservoir might make it difficult for darters to use a reservoir as a corridor to other populations. All of these reasons may cause populations of darters to become isolated if they are directly connected to a reservoir, and therefore cause the genetic diversity of the populations to decrease.

In order to determine the genetic effects habitat fragmentation has on fish populations, a DNA marker which shows these effects must be chosen. Microsatellite DNA, also known as SSRs (simple sequence repeats) consist of tandem repeats of one to six base pairs (ex. $GATA_4$). These tandem repeats are widely dispersed throughout the genome and occur approximately every 10 kbp (O'Connell and Wright 1997). Microsatellite DNA can detect population isolation occurring in as little as 13 generations, making this type of DNA ideal for short term isolation studies (Hendry et al.

2000). Microsatellite DNA is able to show changes in the genetic structure because of its rapid mutation rate. This rapid mutation rate is due to slippage during DNA replication that is attributed to the repetitive nature of microsatellite DNA. Because microsatellites do not code for protein they are assumed to be selectively neutral (Ellegren, XXX). Microsatellite DNA has been utilized in population studies with diverse research goals in a wide variety of organisms (Paetkau et al. 1997; Brohede et al. 2002; Gum et al. 2003; Saillant et al. 2004; Alò and Turner 2005; Hubert et al. 2008).

The purpose of this study is to determine whether habitat fragmentation caused by Barren River Reservoir is genetically isolating populations of *Etheostoma caeruleum* and *Etheostoma kantuckeense* inhabiting streams adjacent to Barren River Reservoir. If genetic isolation was occurring in stream fish, the more environmentally sensitive species (darters) would be the first to provide evidence for it. It is hypothesized that the change in habitat created by Barren River Reservoir has isolated populations of darters living in streams adjacent to the reservoir. Microsatellite DNA is utilized to test the null hypothesis that there would not be any genetic differences between populations in streams connected to the reservoir and those in the free-flowing sections of Barren River drainage upstream of the reservoir.

Materials and Methods

Study Area and Sample Collection

Individuals of *Etheostoma caeruluem* and *Etheostoma kantuckeense* were collected from 6 tributaries of the Barren River Drainage in the summer of 2008 (Rhoden Creek, Walnut Creek and Peter Creek directly connected to Barren River Lake; Puncheon Creek, Salt Lick Creek and Indian Creek directly connected to Barren River upstream of the reservoir) (Figure 1). Barren River Reservoir was completed in 1964 with the primary purposes of flood control and for storm water management in South Central Kentucky. The reservoir drains 1,512 km^2 above Barren River Dam, has a length of 1,210 m and a width of 387 m (US Army Corps of Engineers, 2009). All individuals were collected using backpack electrofishing methods, then placed in 75% ethyl alcohol (EtOH) and stored at -20^oC prior to DNA extraction. Total genomic DNA was extracted from the caudal peduncle muscle tissue using the QIAGEN™ DNeasy™ DNA extraction kit. All DNA samples were stored in a -20^oC freezer until analysis. Following DNA extraction, all fish were fixed in formalin and returned to 75% EtOH for long term storage.

DNA amplification

Three microsatellite DNA loci (Eca10EPA, Eca11EPA, and Eca44EPA) (Tonnis 2006) were amplified using a BioRad™ MyCycler Thermocycler in a 25 ul reaction mixture containing 12 ul Nanopure deionized distilled H_2O, 10 ul 2.5x PCR MasterMix (5Prime) and 1 ul each of template DNA, fluorescently labeled forward primer, and

unlabeled reverse primer (Table 1). Polymerase chain reaction (PCR) conditions were as follows: 95°C for 1 min; 12 cycles of (95°C for 30 s, 64°C for 30 s, dropping 0.8°C per cycle after the initial cycle, 72°C for 1.5 min); 23 cycles of (95°C for 30 s, 54°C for 30 s, 72°C for 1.5 min); final extension of 72°C for 15 min and hold at 4°C (Tonnis 2006). Samples were analyzed using an Applied Biosystems ™ ABI 3130 Genetic Analyzer fitted with a 50 cm capillary array. Amplified alleles and their respective sizes were determined using GENEMAPPER version 3.7 (Applied Biosystems) using the following specifications for each microsatellite locus: analysis range of 95-500 base pairs, minimum peak detection height of 1000, and a bin width of 4 base pairs. Alleles identified by GENEMAPPER were confirmed by manual examination of each run.

Statistical Analysis

GENEPOP software (Raymond and Rousset, 1995) was utilized to determine conformity to Hardy-Weinberg equilibrium, as well as basic population genetic statistics such as observed (H_O) and expected heterozygosity (H_E), total number of alleles (N) and allele frequencies at each locus.

Effective number of alleles (A_e) was also determined for each population. The effective number of alleles is a descriptive statistic that allows for comparison between populations normalizing for differences in population size. A_e is estimated by:

$$A_e = 1/\sum p_i^2$$

where p_i is the frequency of a given allele. Low values for A_e in relation to N (total number of alleles), may indicate low genetic diversity for a population (Hartl and Clark,

1997). A two-tailed t-test was utilized to determine whether there were significant differences in N, H_o, and A_e at river sites versus reservoir sites for each species.

Isolation-by-distance

In order to determine whether geographic distance between populations is causing genetic isolation, GENEPOP software was utilized using the ISOLDE subprogram option by Mantel test with 1000 permutations. This test indicates how much of a factor distance plays in the isolation of populations of *E. caeruleum* and *E. kantuckeense*.

Private Alleles

Private Alleles are identified as alleles that are unique to a particular population within the dataset for one species. For the purposes of this study, private alleles were determined from allele frequency data (Appendix 1) in an attempt to determine whether exchange of alleles occurs across the species barrier. Private alleles were also utilized to determine whether there are alleles that are specific to only reservoir or only river sites within each species.

Within-site variation

The M-ratio for each population was calculated according to Garza and Williamson (2001) in order to determine whether a population bottleneck has occurred within any of the study populations. Calculated as $M = k/r$, where k is the total number of alleles found at a population for a locus and r is the overall range in allele size, M-ratio is based on the idea that if a population has experienced a bottleneck, and subsequent genetic drift, then the number of alleles present in the population will drop before a

decrease in allele size range will occur (Garza and Williamson, 2001). Because the loss of alleles due to genetic drift is stochastic and rare alleles are usually dispersed among other alleles (i.e. not the largest or smallest alleles), then the total number of alleles found in a population will drop faster than the range of alleles at the same site. This would cause the *M* to decrease. Values of M less than 0.7 are interpreted as indicating a recent bottleneck event in the population (Garza and Williamson, 2001).

Results

A total of 125 *Etheostoma caeruleum* were collected from the 6 study sites, and 74 *Etheostoma kantuckeense* were collected from 4 study sites (*E. kantuckeense* were not collected from Puncheon Creek or Peter Creek). The number of fish collected per site ranged from 10 to 26 for *E. caeruleum* and 10 to 25 for *E. kantuckeense*. All three microsatellite loci (eca10EPA, eca11EPA and eca44EPA) were successfully amplified for all individuals of *Etheostoma caeruleum*. For *Etheostoma kantuckeense*, however, all individuals were successfully amplified for only two of the three loci (eca10EPA and eca11EPA) (Table 1). Locus eca44EPA consistently failed to amplify for individuals of *Etheostoma kantuckeense*. Therefore, any analyses referring to *E. kantuckeense* includes only data collected from loci eca10EPA and eca11EPA.

Test for deviations from Hardy-Weinberg Equilibrium (HWE) revealed only 7 of 26 total populations in equilibrium (*E. caeruleum:* 3 loci and six study sites = 18 total populations; *E. kantuckeense:* 2 loci and 4 study sites = 8 total populations). Deviations from HWE are due to heterozygote deficiencies.

Within-site variance for *Etheostoma caeruleum*

The three loci successfully amplified for *Etheostoma caeruleum* yielded 7-16 alleles per site, as well as an average observed heterozygosity of 0.875 ±0.022 (Table 2). Effective number of alleles (A_e) ranged from 5.9 to 12.6 alleles, but were not found to be significantly different between river and reservoir sites at any of the three loci (eca10EPA, p= 0.84; eca11EPA, p= 0.48; eca44EPA, p= 0.58). T-test results also

revealed that there was no significant difference between river and reservoir sites when comparing total number of alleles (N) (eca10EPA, p= 0.69; eca11EPA, p=0.38; eca44EPA, p= 1) and observed heterozygotsity (H_o) (eca10EPA, p= 0.36; eca11EPA, p=0.56; eca44EPA, p=0.34) at any locus. *M*-ratios ranged from 0.49 to 1, suggesting that, overall, all of the study populations of *Etheostoma caeruleum* have not been subjected to a bottleneck event in recent history (Table 3).

Within-site variance for *Etheostoma kantuckeense*

Two successfully amplified loci for *Etheostoma kantuckeese* yielded 2-16 alleles per site and an average observed heterozygosity of 0.783 ± 0.161 (Table 4). A_e for all sites ranged from 1.98 to 9.8 and, as seen in *E. caeruleum*, was not found to be statistically significant at either locus when compared by t-test between river and reservoir populations (eca10EPA, p= 0.17; eca11EPA, p= 0.27). T-test results agreed with patterns seen in *E. caeruleum* in that neither N (eca10EPA, p= 0.29; eca11EPA, p= 0.54) nor H_o (eca10EPA, p= 0.1; eca11EPA, p= 0.97) were found to be significantly different in river and reservoir sites at any locus. Average *M*-ratios seen in *E. kantuckeense* populations ranged from 0.27 to 0.62 (Table 3).

Isolation by distance

The Mantel test (1000 permutations) performed using GENEPOP detected no influence of geographic distance between populations on the genetic differences among populations. The p-values for the comparison on *E. caeruleum* DNA at each locus were as follows: eca11EPA- p = 0.15, eca10EPA- p = 0.29, eca44EPA- p = 0.54). These p-values indicated that populations of *E. caeruleum* are not genetically isolated due to the

geographic distance between them. The same was true for populations of *Etheostoma kantuckeense* (eca11EPA- p = 0.56, eca10EPA- p = 0.37). This test indicated that any genetic differences seen within either species is not due to the geographic distances between the respective populations.

Private Alleles

A large number of private alleles (alleles seen at one site within a species dataset) exist within the dataset. A total of 26 private alleles at three amplified loci occur within populations of *E. caeruleum*, and 29 private alleles exist at two amplified loci within populations of *E. kantuckeense*. All private alleles, with the exception of one present at Salt Lick Creek (frequency= 0.45), occur at a frequency of less than 0.16. Of the 55 total private alleles, 7 of those are either shared between species at a single site (i.e., only at Indian Creek) or present at one site in *E. kantuckeense* and many sites in *E. careuleum* (or vise versa). There does not seem to be a trend towards the presence of alleles being shared at reservoir sites over river sites in *E. caeruleum*, but there are 4 alleles seen only at reservoir sites in *E. kantuckeense*. Private alleles that are shared between species occur at a frequency less than 0.15 at each site.

Discussion

Analysis of microsatellite DNA did not detect differences among populations of *E. caeruleum* and *E. kantuckeense* in the Barren River Drainage, regardless of the population's position relative to Barren River Reservoir. The null hypothesis stating there would be no genetic differences between populations in streams connected to the reservoir and those in the free-flowing sections of Barren River drainage upstream of the reservoir was not rejected.

Many studies have determined that distance plays an important factor in the genetic isolation of fishes (Pogson et al., 2001; Castric and Bernatchez 2003). This study, however, did not detect any influence of distance on genetic diversity between populations. This may be due to the relatively short distances between sites used for this study (maximum distance= 65.47 river kilometers), while the previously mentioned studies utilized distances upwards of 7,000 km between populations.

When populations were tested for deviations from Hardy-Weinberg equilibrium, it was discovered that only 7 out of 24 populations were in equilibrium. The populations that were not found to be in equilibrium deviated due to heterozygote deficiencies. Heterozygote deficiencies have caused HWE deviations in other studies involving members of the *Etheostoma* genus (Johnson et al., 2006), but not to the extent seen here.

Private alleles seen within the dataset, with the exception of one private allele present in the Salt Lick Creek population of *E. kantuckeense* (frequency = 0.45), all occur at frequencies of less than 0.16. What is of particular interest is the relatively high number of private alleles that are shared across the species barrier, which could indicate

two things: 1) These alleles could have arisen independently through random mutation events, or 2) *Etheostoma caeruleum* and *E. kantuckeense* may be hybridizing and sharing alleles. Hybridization events involving *Etheostoma caeruleum* and *Etheostoma spectabile* (the species from which *E. kantuckeense* was described) have been documented to occur with other species of darter in natural settings (Branson and Campbell, 1969; Ray et al., 2008). Two alleles are shared at only one site between the species, and five alleles are found at one site in *E. kantuckeense* (or *E. caeruleum*) and found at most sites in the other species. All shared private alleles are found at Rhoden Creek (reservoir site) and Indian Creek (river site). These sites are 19.5 river kilometers apart. This could be evidence for the occurrence of gene flow between Rhoden and Indian Creeks, but it does not explain why these alleles are only found at these two sites because there is a river site that is geographically situated between these sites. One explanation is that these alleles were once present at other sites, but have been eliminated by some evolutionary process, such as genetic drift. The low frequencies at which these alleles exist supports this hypothesis.

Levels of observed heterozygosity (H_o), effective number of alleles (A_e), and total number of alleles (N) were relatively high across all sites and did not differ within each species between river sites and reservoir sites (Table 2 for *Etheostoma caeruleum*; Table 3 for *Etheostoma kantuckeense*). Specimens of *E. kantuckeense* from Salt Lick Creek (river site) showed a different pattern from what was observed at all other locations. H_o at this site was the lowest seen across all other study sites at locus eca11EPA (H_o=0.1). N was also the lowest at this site (N=5 across both amplified loci). Overall, these parameters do not indicate the reservoir having an adverse effect on either of these

species. Each species is showing the ability to maintain high genetic diversity at all sites, regardless of its position on Barren River Lake or upstream on Barren River.

It was observed during the study that a difference in microhabitat preference existed between populations of *E. caeruleum* and *E. kantuckeense* inhabiting Salt Lick Creek. No specimens of *E. kantuckeense* were initially collected from the mainstem of Salt Lick Creek even though many individuals of *E. caeruleum* were present. At a later date, the site was resampled and a fairly large population of *E. kantuckeense* was found to inhabit a small tributary no more than 30 m from where Salt Lick Creek was sampled initially, and a large difference in stream width and stream depth existed between the mainstem of Salt Lick Creek and the small tributary. If *E. kantuckeense* prefers smaller streams, it would be reasonable to conclude that it would be less likely to make a migratory trip from one population to another if sections of large river habitat (or reservoir habitat) exist between them. This would help explain the lower genetic variability seen at Salt Lick Creek (Table 4).

With the patterns seen in both species showing that there is no difference in H_o, A_e, and N between river and reservoir sites, it is the conclusion of the author that the reservoir does not seem to be effecting populations of *Etheostoma caeruleum* and *Etheostoma kantuckeense* by serving as a habitat barrier. This conclusion is further supported by patterns seen in *M*-ratios. *M*-ratios can be used to indicate a recent bottleneck occurring in a population. When *M* is low, it indicates that a bottleneck has occurred recently, and can take up to 125 generations to rebound to 90% of that population's original *M* (Garza and Williamson, 2001). If the reservoir is acting as a

barrier to gene flow and isolating populations, or had caused a bottleneck to occur, we would see lower *M* at reservoir adjacent sites when compares to river adjacent sites. M-ratios for *Etheostoma caeruleum* indicate that a bottleneck has not occurred to these populations, with all *M*'s falling above the 0.7 threshold (See Table 4). One exception is Rhoden Creek (*M*= 0.499). This site exhibits the smallest sample size of all *E. caeruleum* populations (n= 10), indicating that sample size may be related to the calculated *M*-ratio of a population. A larger sample size for this population is needed in order to determine whether the calculated *M* is true for this population.

M-ratios for *Etheostoma kantuckeense* show the opposite trend of *E. caeruleum*, indicating that a bottleneck has occurred to all study populations (See Table 4). As seen in *E. caeruleum*, sample size seems to play an important role in determining the *M* of a population. Although not above the 0.7 threshold, Rhoden Creek (n= 25) exhibits the highest *M* across all *E. kantuckeense* populations (*M*= .623). This provides further evidence for the correlation of *M*-ratios with sample size.

When a bottleneck occurs in a population, one would expect the allelic diversity and *M*-ratio of that population to decrease. The *M*-ratio for *Etheostoma kantuckeense* suggests that bottleneck has occurred, but high allelic diversity is seen at most sites (the exception being Salt Lick Creek). This seemingly contradicting data may suggest that all of the study populations are rebounding from a bottleneck that occurred in the last 125 years. This data trend (low *M*, high allelic diversity) could occur because the small sample sizes used in this study failed to detect some alleles in the natural population. It could also occur because of the nature of microsatellite alleles. Rare alleles are not

usually found as outliers, so as new alleles are being introduced into a population, they are most likely being introduced into the middle of the range of alleles at a given locus. This would increase the number of alleles in a population before an increase in range is seen, causing *M* to remain low while allelic diversity is high (Garza and Williamson, 2001).

Three hypotheses exist for the cause of the bottleneck effect that is evident in populations of *Etheostoma kantuckeese*. First, Barren River Dam, which was built in 1964, could be acting as a physical barrier to migrants from downstream populations. Although the migration patterns of *Etheostoma kantuckeense* are unknown, if they are normally able to travel upstream in a large river they could be blocked from their normal migration routes, causing genetic drift to occur. This blocking of migration has been documented in many other fish (Neeras and Spruell, 2001; Morita and Yamamoto, 2002; Meldgaard et al., 2003; Yamamoto et al., 2004). If the blocking of migrants from downstream is adversely effecting populations of *E. kantuckeense*, one would expect the same effect to be evident in *Etheostoma caeruleum* as well, but this is not being shown in the data. Second, the reservoir itself may have affected all *E. kantuckeense* populations in a negative fashion. A study on the creek chub (*Semotilus atromaculatus*) showed that populations inhabiting tributaries flowing directly into a reservoir had markedly less genetic diversity than those populations inhabiting tributaries to the river (Skalski et al., 2008). While this does not agree with the findings of this study (lower genetic diversity across all study populations of *Etheostoma kantuckeense*), it does show that reservoirs affect some species of fish. *Etheostoma kantuckeense* (along with *E. caeruleum*) is considered a headwater species like *Semoltilus atromaculatus*, but *E. kantuckeense* may

be more sensitive to changes within its environment, even more so than *E. caeruleum*. The effects of small changes within a river that has been impounded (temperature, dissolved oxygen, increased sedimentation) may be affecting darters (specifically *E. kantuckeense*) farther upstream than many other species of stream fish. This hypothesis could be further supported by the difference in microhabitat preference seen at Salt Lick Creek. If individuals of *E. kantuckeense* are not able to inhabit a larger section of river, then the mainstem of Barren River, along with Barren River Reservoir could be acting as a genetic barrier to *Etheostoma kantuckeense*. A selection of sites along an unimpounded stretch of river far from an impoundment could determine how far upstream the reservoir's effects are seen, as well as selecting tributaries that are separated by sections of river of different stream order. This could provide insight into the habitat preference of *E. kantuckeense*, as well as whether or not an unimpounded stretch of river actually acts as a genetic isolating mechanism to this species. Finally, there have been persistent droughts affecting the study area over the past few decades. If these droughts were severe enough, they could have caused bottlenecks to occur in all of the study populations. The trend seen in the *M*-ratios for populations of *Etheostoma kantuckeense* indicate that these populations are on the rebound from a bottleneck that occurred in the last 100 years. A particularly severe drought that occurred in the 1920's could have cause the initial bottleneck for these populations, and all other factors (reservoir and dam construction, and recent persistent droughts) could have served as smaller bottlenecks. One would expect a severe drought to affect all species of fish, but environmentally sensitive species of fish (such as *Etheostoma kantuckeense*) may show the effects for a longer period of time, especially in the light of the recent and persistent droughts.

Populations of *Etheostoma caeruleum* inhabiting tributaries adjacent to Barren River Reservoir seem to be as genetically diverse as populations inhabiting tributaries to Barren River upstream of the dam. There also seems to be no difference in the genetic diversity of populations of *Etheostoma kantuckeense* inhabiting tributaries adjacent to Barren River Reservoir and Barren River, but unlike *E. caeruleum*, all populations of *E. kantuckeense* seem to have gone though a population bottleneck in recent history. The scope of this study cannot definitively say what has caused this bottleneck, but can only speculate events that may have caused it. But it does seem that *Etheostoma kantuckeense* is more sensitive to its environment as suggest by a distinct difference in microhabitat preference seen at Salt Lick Creek. If *E. kantuckeense* is more sensitive to its environment, it would be beneficial for those wanting to conserve the native fish populations that inhabit South Central Kentucky. When conservation agencies assess fish populations in South Central Kentucky, it would be advantageous for them to know which species are already at risk, which species are sensitive to changes in their environment, and which species or populations are recovering from events that were detrimental to their genetic diversity. Knowing which species or populations fit into these categories can help them make the best conservation decisions to benefit stream fish.

Figures

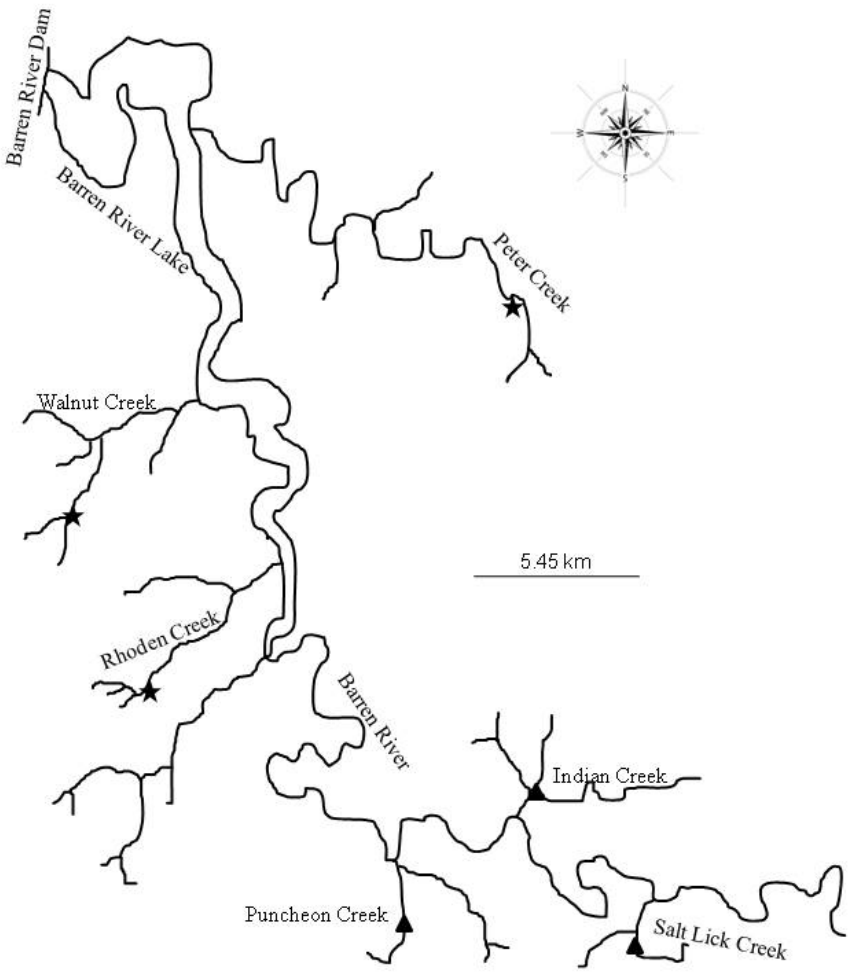

Figure 1. Diagram showing each study site in relation to Barren River Reservoir and Barren River. Sites labeled with a solid star are sites that are reservoir-adjacent, and sites labeled with a solid triangle are river-adjacent sites.

Tables

Table 1. Primer sequence and summary statistics for microsatellite loci utilized in *Etheostoma caeruleum* and *Etheostoma kantuckeense* (Toonis, 2006).

Locus	Accession Number	Primer sequence (5'-3')	Expected Range (bp)
eca10EPA	DQ205700	**F**:TGCACAATGAAGTTAAGATGCTGT	204-282
		R:GAATGGCTCTGATATTGCATGATT	203-244
eca11EPA	DQ205699	**F**:CGGGCCAGGTTGGTTTAAATG	198-292
		R:GCAGAAGCACAGGAAAGCACCCCCTCAA	151-297
eca44EPA*	DQ205692	**F**:AATGTTGCTGACGCAGATTGTA	138-178
		R:ACTGGGACCATGAATTTCCA	132-254

* locus not amplified for individuals of *Etheostoma kantuckeense*

Table 2. Basic Population statistics for *Etheostoma caeruleum*. Includes total number of alleles (N), observed heterozygosity (H_o), and effective number of alleles (A_e) for each locus at each study site. The symbol (n) denotes the number of fish whose DNA was successfully amplified for each loci at each site.

Locus		Puncheon Cr. river	Salt Lick Cr. river	Indian Cr. river	Rhoden Cr. reservoir	Walnut Cr. reservoir	Peter Cr. reservoir	Overall
eca11EPA		n=25	n=20	n=20	n=10	n=26	n=24	n=125
	N	15	11	12	15	16	12	27
	H_o	0.88	0.95	0.95	0.9	1	0.95	0.94
	A_e	10	7.7	7.8	9.5	12.6	7.3	
eca10EPA		n=25	n=20	n=20	n=10	n=26	n=23	n=124
	N	12	9	14	11	13	13	24
	H_o	0.88	0.4	0.6	0.8	0.85	0.69	0.7
	A_e	8.9	6.2	10	8.3	9	7	
eca44EPA		n=25	n=20	n=20	n=10	n=26	n=23	n=124
	N	9	10	12	7	12	12	18
	H_o	0.84	0.7	1	0.6	0.77	0.82	0.8
	A_e	6.6	6.3	8.4	5.9	6.7	8.5	

Table 3. *M*-ratios for both *Etheostoma caeruleum* (top value) and *Etheostoma kantuckeense* (bottom value) at all study sites, across all amplified loci.

	eca10EPA	eca11EPA	eca44EPA	Average
Puncheon Creek	1	1	1	1
Salt Lick Creek	0.82 0.214	0.647 0.33	1	0.822333 0.272
Rhoden Creek	0.46 0.517	0.5 0.73	0.538	0.499333 0.6235
Indian Creek	0.56 0.32	0.8 0.58	0.923	0.761 0.45
Walnut Creek	1 0.714	0.84 0.31	1	0.946667 0.512
Peter Creek	1	0.8	0.75	0.85

Table 4. Basic Population statistics for *Etheostoma kantuckeense*. Includes total number of alleles (N), observed heterozygosity (H_o), and effective number of alleles (A_e) for each locus at each study site. The symbol (n) denotes the number of fish whose DNA was successfully amplified for each loci at each site.

Locus	Salt Lick Cr. river	Indian Cr. river	Rhoden Cr. reservoir	Walnut Cr. reservoir	Overall
eca11EPA	n=10	n=19	n=25	n=19	n=73

	N	2	15	16	11	29
	H o	0.1	0.63	0.56	0.15	0.41
	A e	1.98	7.35	9.8	7.9	
eca10E PA		n=9	n=19	n=25	n=18	n=71
	N	3	10	15	10	20
	H o	0.66	0.52	0.84	0.78	0.72
	A e	2.26	5.7	9.71	7.2	

LITERATURE CITED

Alò, D., and T. F. Turner. 2005. Effects of habitat fragmentation on effective population size in the endangered Rio Grande silvery minnow. Conservation Biology 19(4):1138-1148.

Anderson, T. J. C., B. Haubold, J. T. Williams, J. G. Estrada-Francos, L. Richardson, R. Mollinedo, M. Bockarie, J. Mokili, S. Mharakurwa, N. French, J. Whitworth, I. D. Velez, A. H. Brockman, F. Nosten, M. U. Ferreria, and K. P. Day. 2000. Microsatellite Markers Reveal a Spectrum of Population Structures in the Malaria Parasite *Plasmodium falciparum*. Molecular Biology and Evolution 17:1467-1482.

Baxter, R. M. 1977. Environmental effects of dams and impoundments. Annual Review of Ecological Systematics 8: 255-283.

Branson, B. A., and J. B. Campbell. 1969. Hybridization in the darters *Etheostoma spectabile* and *Etheostoma radiosum cyanorum*. Copeia (1):70-75.

Ceas, P. A. and L. M. Page. 1997. Systematic studies of the *Etheostoma spectabile* complex (Percidae; Sungenus *Oligocephalus*), with descriptions of four new species. Copeia 1997(3):496-522.

Distler, D. 1968. Distribution and variation of *Etheostoma spectabile* (Agassiz) (Percidae, Teleostei). University of Kansas Scientific Bulletin 48:143-208.

Doerner, K. C., W. Braden, J. Cork, T. Cunningham, A. Rice, B. J. Furman, and D. McElroy. 2005. Population genetics of resurgence: White-tailed deer in Kentucky. The Journal of Wildlife Management 69(1):345-355.

Dynesius, M., and C. Nilsson. 1994. Fragmentation and flow regulation of river systems in the Northern third of the world. Science 266(5186): 753-762.

Echelle, A. A., A. F. Echelle, M. H. Smith, and L. G. Hill. 1975. Analysis of genic continuity in a headwater fish, *Etheostoma radiosum* (Percidae). Copeia 1975:197-204.

Falke, J. A., and K. B. Gido. 2006. Effects of reservoir connectivity on stream fish assemblages in the Great Plains. Canadian Journal of Fishery and Aquatic Science 63:480-493.

Garza, J. C., and E. G. Williamson. 2001. Detection of reduction in population size using data from microsatellite loci. Molecular Ecology 10:305-318.

Gum, B., R. Gross, O. Rottman, W. Schröder, and R. Kühn. 2003. Microsatellite variation in Bavarian populations of Eurpoean grayling (*Thymallus thymallus*): implications for conservation. Conservation Genetics 4:659-672.

Heithaus. M. R. and R. H. Laushman. 1997. Genetic variation and conservation of stream fishes: Influence of ecology, life history, and water quality. Canadian Journal of Fisheries and Aquatic Sciences 54:1822-1836.

Hendry, A. P., J. K. Wenburg, P. Bentzen, E. C. Volk, and T. P. Quinn. Rapid Evolution of Reproductive Isolation in the Wild: Evidence from Introduced Salmon. Science 290(5491):516-518.

Hutchinson, W. F., G. R. Carvalho, and S. I. Rogers. 2001. Marked genetic structuring in localized spawning populations of cod *Gadus marhua* in the North

Sea and adjoining waters, as revealed by microsatellites. Marine Ecology Progress Series 223:251-260.

Johnson, R. L., R. M.Mitchell, and G. L. Harp. 2006. Genetic variation and genetic structuring of a numerically declining species of darter, *Etheostoma moorei* Raney and Suttkus, endemic to the Upper Little Rid River, Arkansas. American Midland Naturalist 156:37-44.

Lienesch, P., W. Lutterschmidt, and J. Schaefer. 2000. Seasonal and Long-Term Changes in Fish Assemblage of a Small Stream Isolated by a Reservoir. The Southwestern Naturalist 45(3):274-288.

Lincoln, R., G. Boxwell, and P. Clark. 2003. A Dictionary of Ecology, Evolution and Systematics: Second Edition. Cambridge: Cambridge University Press.

MacArthur, R. H., and E. O. Wilson. 1963. An Equilibrium theory of insular zoogeography. Evolution 17(4):373-387.

Martinez, P. J., T. E. Chart, M. A. Trammell, J.G. Wullschleger, and e. P. Bergersen. 1994. Fishe species composition before and after construction of a main stem reservoir on the White River, Colorado. Environmental Biology of Fishes 40:227-239.

Matthews, W. J., and E. Marsh-Matthews. 2007. Extirpation of Red Shiner in Direct Tributaries of Lake Texoma (Oklahoma-Texas): A Cautionary Case History from a Fragmented River-Reservoir System. Transactions of the American Fisheries Society 136:1041-1062.

Meldgaard, T., E. E. Nielsen, and V. Loeschcke. 2003. Fragmentation by weirs in a riverine system. A study of genetic variation in time and space among populations of European grayling (*Thymallus thymallus*) in a Danish river system. Conservation Genetics 4:735-747.

Neraas, L. P., and P. Spruell. 2001. Fragmentation of Riverine Systems: The Genetic Effects of Dams on Bull Trout (*Salvelinus confluentus)* in the Clark Fork River. Molecular Ecology System 10:1153-1164.

O'Connell, M., and J. M. Wright. 1997. Microsatellite DNA in fishes. Reviews in Fish Biology and Fisheries 7:331-363.

Paetkau, D., W. Calvert, I. Stirling, and C. Strobeck. 1995. Microsatellite analysis of population structure in Canadian Polar Bears. Molecular Ecology 4(3):347-354.

Pflieger, W. L. 1997. The Fishes of Missouri. Missouri Department of Conservation, Jefferson City.

Phillips, B. W. and C. E. Johnston. 2004. Changes in the fish assemblage of Bear Creek (Tennessee River Drainage) Alabama and Mississippi: 1968-2000. Southeastern Naturalist 3(2):205-218.

Pogson, G. H., C. T. Taggart, K. A. Mesa, and R. G. Boutilier. 2001. Isolation by distance in the Atlantic cod, *Gadus morhua*, at large and small geographic scales. Evolution 55(1):131-146.

Raven, P. H., G. B. Johnson, J. B. Losos, K. A. Mason, and S. R. Singer. 2008. Biology. McGraw Hill, New York.

Ray, J. M., R. M. Wood, and A. M. Simmons. 2006. Phylogeography and post-glacial colonization patterns of the rainbow darter, *Etheostoma caeruleum* (Teleostei: Percidae). Journal of Biogeography 2006(33): 1550-1558.

Ray, J. M., N. J. Lang, R. M. Wood, and R. L. Mayden. 2008. History repeated: recent and historical mitochondrial introgression between the current darter *Etheostoma uniporum* and rainbow darter *Etheostoma caeruleum* (Teleostei: Percidae). Journal of Fish Biology 72(2):418-434.

Raymond M. & Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity, 86:248-249

Saillant, E., J.C. Patton, K. E. Ross, J. R. Gold. 2004. Conservation genetics and demographic history of the endangered Cape Fear shiner (*Notropis mekistocholas*). Molecular Ecology 13(10):2947-2958.

Shelton, W. L. and W. D. Davies. 1977. Preimpoundment survey of fishes in the West Point Reservoir area (Chattahoochee River, Alabama and Georgia). Georgia Journal of Science 35:221-230.

Shelton, W. L., and W. D. Davies. 1981. West Point Reservoir- A Recreation Demonstration Project. In: Stefan, H. G., editor. Proceedings of the symposium on surface water impoundments. American Society of Civil Engineers, Minneapolis, Minnesota. Pp. 1419-1431.

Skalski, G. T., J. B. Landis, M. J. Grose, and S. P. Hudman. 2008. Genetic structure of a headwater minnow in an impounded river system. Transactions of the American Fisheries Society 137(4):962-975.

Stallings, R. L. 1995. Conservation and evolution of $(CT)_n/(GA)_n$ microsatellite sequences at orthologous positions in diverse mammalian genomes. Genomics 25(1):107-113.

Timmons, T. J., W. L. Shelton, and W. D. Davies. 1978. Initial fish population changes following impoundment of West Point Reservoir, Alabama-Georgia. Proceedings of the Annual Conference of the Southeastern Association of Fisheries and Wildlife Agencies 31:312-317.

Tonnis, B. D. 2006. Microsatellite DNA markers for the rainbow darter, *Etheostoma caeruleum* (Percidae), and their potential utility for other darter species. Molecular Ecology Notes 6:230-232.

Turner, T. F. and J. C. Trexler. 1998. Ecological and historical associations of gene flow in darters (Teleostei: Percidae). Evolution 52(6):1781-1801.

US Army Corps of Engineers. 2009. Barren River Lake Purpose and Facts. Retrieved from US Army Corps of Engineers Website: http://www.lrl.usace.army.mil/brl/article.asp?id=282&MyCategory=1.

Yamamoto, S., K. Morita, I. Koizumi, and K. Maekawa. 2004. Genetic differentiation of white-spotted char (*Salvelinus leucomaenis*) populations after habitat fragmentation: Spatial-temporal changes in gene frequencies. Conservation genetics 5:529-538.

APPENDIX I

Appendix 1. Allele frequency data for all sites. Private Alleles indicated with a (*), shared private alleles in bold face type.

Species	Locus	Puncheon Creek		Salt Lick Creek		Rhoden Creek		Indian Creek		Walnut Creek		Peter Creek	
		Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
Etheostoma caeruleum	eca10EPA	200	0.08	208	0.15	164	0.1	164	0.025	200	0.019	204	0.043
		208	0.16	212	0.225	***180**	0.1	***196**	0.05	204	0.057	208	0.065
		212	0.16	216	0.225	*192	0.05	200	0.025	208	0.038	216	0.043
		218	0.04	220	0.05	208	0.15	208	0.175	212	0.096	218	0.021
		220	0.04	228	0.1	216	0.05	212	0.1	216	0.038	220	0.021
		*222	0.12	232	0.15	220	0.2	216	0.1	220	0.076	224	0.021
		224	0.02	236	0.025	224	0.05	224	0.15	224	0.134	228	0.26
		228	0.08	244	0.025	228	0.05	228	0.05	228	0.115	232	0.195
		232	0.18	*246	0.025	240	0.15	232	0.05	232	0.134	236	0.152
		236	0.04			256	0.05	236	0.05	236	0.192	240	0.043
		240	0.04			*260	0.05	240	0.075	*238	0.019	244	0.086
		244	0.04					244	0.05	240	0.057	*252	0.021
								256	0.075	244	0.019	256	0.021
								*264	0.025				

ec11EPA	179	0.08	*173	0.05	179	0.1	***175**	0.075	*177	0.135	*181	0.063
	185	0.08	185	0.1	185	0.1	179	0.025	185	0.077	185	0.021
	189	0.12	189	0.15	189	0.1	185	0.075	189	0.115	193	0.042
	193	0.16	193	0.15	193	0.1	189	0.175	193	0.096	197	0.104
	197	0.1	197	0.025	197	0.05	193	0.1	197	0.058	201	0.25
	201	0.04	201	0.225	201	0.05	197	0.225	201	0.038	205	0.146
	205	0.16	205	0.05	205	0.05	201	0.1	205	0.115	209	0.083
	209	0.04	209	0.1	213	0.05	205	0.05	209	0.058	213	0.146
	*211	0.02	217	0.025	217	0.1	213	0.025	213	0.038	217	0.083
	213	0.06	225	0.075	225	0.1	217	0.1	217	0.058	229	0.021
	217	0.04	***235**	0.05	229	0.1	225	0.025	221	0.019	237	0.021
	221	0.04			237	0.1	229	0.025	225	0.058	241	0.021
	*227	0.02			245	0.05			233	0.038		
	233	0.02			***284**	0.05			241	0.019		
	237	0.02			***298**	0.1			245	0.019		
									***253**	0.019		
eca44EPA	124	0.16	124	0.102	116	0.05	116	0.025	124	0.057	116	0.043

		128	0.02	128	0.256	*122	0.25	*120	0.025	128	0.019	124	0.021
		132	0.1	132	0.025	*130	0.2	124	0.1	132	0.038	128	0.043
		136	0.1	136	0.179	136	0.15	128	0.075	136	0.115	136	0.173
		140	0.2	140	0.153	144	0.15	132	0.075	140	0.288	140	0.173
		144	0.14	144	0.057	148	0.1	136	0.175	144	0.134	144	0.065
		148	0.2	148	0.128	168	0.1	140	0.125	148	0.096	148	0.152
		152	0.06	152	0.051			144	0.15	*150	0.019	152	0.108
		156	0.02	156	0.025			148	0.15	152	0.019	160	0.086
				160	0.051			152	0.025	156	0.134	*162	0.043
								156	0.025	160	0.038	168	0.065
								168	0.05	*164	0.038	*180	0.021
Etheostoma kantuckeense	eca10EPA			*148	0.166	*124	0.02	*132	0.054	180	0.222		
				196	0.555	*154	0.02	196	0.108	*188	0.138		
				204	0.277	***164**	0.04	208	0.351	200	0.111		
						***180**	0.02	212	0.054	204	0.194		
						196	0.02	216	0.081	208	0.027		
						200	0.14	220	0.054	212	0.083		

			208	0.06	224	0.108	216	0.083
			212	0.14	228	0.081	220	0.055
			216	0.08	232	0.081	224	0.027
			220	0.02	***256**	0.027	236	0.055
			224	0.12				
			228	0.06				
			232	0.02				
			236	0.16				
			***240**	0.08				
eca11EPA	*167	0.45	*175	0.02	*151	0.027	*159	0.135
	189	0.55	185	0.02	***175**	0.027	185	0.135
			189	0.08	***179**	0.054	189	0.135
			197	0.2	*183	0.027	201	0.216
			201	0.1	189	0.027	205	0.027
			205	0.1	197	0.027	***235**	0.054
			209	0.02	201	0.189	267	0.054
			213	0.04	205	0.162	*271	0.054

217	0.04	209	0.243	***284**	0.108
***221**	0.08	213	0.027	*293	0.027
229	0.12	217	0.027	***297**	0.054
***233**	0.08	229	0.054		
***237**	0.02	242	0.027		
242	0.02	*249	0.054		
***245**	0.04	***253**	0.027		
*262	0.04				

APPENDIX II

Appendix 2. Data containing alleles called for each individual analyzed. Data is input ready for GENEPOP software. Individual codes are Site number (1-Puncheon creek; 2-Salt Lick Creek; 3-Rhoden Creek; 4-Indian Creek; 5-Walnut Creek; 6-Peter Creek) -Species code (*Etheostoma caeruleum*- 1; *Etheostoma kantuckeense*- 2) – individual tag number.

Microsat on Etheostoma
eca11EPA
Pop
1-1-1, 193205
1-1-2, 189197
1-1-3, 193221
1-1-4, 217227
1-1-5, 201205
1-1-6, 189189
1-1-7, 193205
1-1-8, 189197
1-1-9, 179217
1-1-10, 193193
1-1-11, 185233
1-1-12, 197205
1-1-13, 185193
1-1-14, 185237
1-1-15, 185211
1-1-16, 193205
1-1-17, 197205
1-1-18, 179213
1-1-19, 197209
1-1-20, 205205
1-1-21, 179209
1-1-22, 193213
1-1-23, 189201
1-1-24, 179213
1-1-25, 189221
Pop
2-1-1, 185201
2-1-2, 189225
2-1-3, 201205
2-1-4, 193209
2-1-5, 173225
2-1-6, 185205
2-1-7, 189193
2-1-8, 201201
2-1-9, 189209
2-1-10, 193217
2-1-11, 173235
2-1-12, 193201
2-1-13, 185209
2-1-14, 193201
2-1-15, 189209
2-1-16, 193235
2-1-17, 197201
2-1-18, 201225
2-1-19, 189201
2-1-20, 185189
Pop
3-1-1, 189229
3-1-2, 201205
3-1-3, 197213
3-1-4, 189229
3-1-5, 217225
3-1-6, 179237
3-1-7, 193245
3-1-8, 185217
3-1-9, 245298
3-1-10, 284284
Pop
4-1-1, 197205
4-1-2, 189193
4-1-3, 175205
4-1-4, 189197
4-1-5, 197201
4-1-6, 189193
4-1-7, 197217
4-1-8, 197229
4-1-9, 189225
4-1-10, 197201
4-1-11, 179217
4-1-12, 189193
4-1-13, 175185
4-1-14, 197201
4-1-15, 189193
4-1-16, 197201
4-1-17, 185189
4-1-18, 185213
4-1-19, 217217
4-1-20, 175197
Pop

5-1-1, 177213
5-1-2, 185189
5-1-3, 201217
5-1-4, 185189
5-1-5, 185205
5-1-6, 177197
5-1-7, 189225
5-1-8, 177209
5-1-9, 193205
5-1-10, 197217
5-1-11, 197209
5-1-12, 217245
5-1-13, 177189
5-1-14, 177253
5-1-15, 193205
5-1-16, 189193
5-1-17, 225233
5-1-18, 201241
5-1-19, 177185
5-1-20, 177193
5-1-21, 205225
5-1-22, 205213
5-1-23, 189233
5-1-24, 193205
5-1-25, 205209
5-1-26, 185221
Pop
6-1-1, 181217
6-1-2, 197201
6-1-3, 197201
6-1-4, 201229
6-1-5, 213241
6-1-6, 201217
6-1-7, 201205
6-1-8, 209217
6-1-9, 201205
6-1-10, 197209
6-1-11, 205217
6-1-12, 213213
6-1-13, 201213
6-1-14, 201205
6-1-15, 197201
6-1-16, 197209
6-1-17, 201205
6-1-18, 193213
6-1-19, 181213
6-1-20, 201205
6-1-21, 185213
6-1-22, 201205
6-1-23, 193237
6-1-24, 181209
Pop
2-2-1, 167189
2-2-2, 167167
2-2-3, 189189
2-2-4, 189189
2-2-5, 189189
2-2-6, 167167
2-2-7, 189189
2-2-8, 167167
2-2-9, 167167
2-2-10, 189189
Pop
3-2-1, 197233
3-2-2, 201217
3-2-3, 197197
3-2-4, 174197
3-2-5, 201205
3-2-6, 205237
3-2-7, 205205
3-2-8, 197197
3-2-9, 185197
3-2-10, 189242
3-2-11, 217221
3-2-12, 229233
3-2-13, 229233
3-2-14, 229233
3-2-15, 197201
3-2-16, 201201
3-2-17, 229229
3-2-18, 246246
3-2-19, 189189
3-2-20, 262262
3-2-21, 209221
3-2-22, 205229
3-2-23, 197197
3-2-24, 213213
3-2-25, 221221
Pop
4-2-1, 201201
4-2-2, 205217
4-2-3, 229241
4-2-4, 151179
4-2-5, 175209
4-2-6, 209249
4-2-7, 179183
4-2-8, 209209
4-2-9, 205209
4-2-10, 201201
4-2-11, 205249
4-2-12, 201201
4-2-13, 209253
4-2-14, 189197
4-2-15, 209209
4-2-16, 205205
4-2-17, 201213
4-2-18, 205229
4-2-19, 209209

Pop
5-2-1, 297297
5-2-2, 159205
5-2-3, 185185
5-2-4, 201293
5-2-5, 267267
5-2-6, 283283
5-2-7, 201201
5-2-8, 283283
5-2-9, 185185
5-2-10, 201201
5-2-11, 271271
5-2-12, 159159
5-2-13, 189189
5-2-14, 189201
5-2-15, 159159
5-2-16, 185185
5-2-17, 201201
5-2-18, 189189
5-2-19, 235235

Microsat on Etheostoma
eca10EPA
Pop
1-1-1, 218244
1-1-2, 232232
1-1-3, 224222
1-1-4, 208218
1-1-5, 200208
1-1-6, 212240
1-1-7, 232236
1-1-8, 200208
1-1-9, 212222
1-1-10, 212222
1-1-11, 220232
1-1-12, 212222
1-1-13, 200208
1-1-14, 232244
1-1-15, 212212
1-1-16, 208228
1-1-17, 212222
1-1-18, 220232
1-1-19, 212222
1-1-20, 228240
1-1-21, 200208
1-1-22, 232236
1-1-23, 228232
1-1-24, 228232
1-1-25, 208208
Pop
2-1-1, 208216
2-1-2, 228228
2-1-3, 208216
2-1-4, 216246
2-1-5, 232232
2-1-6, 232236
2-1-7, 220220
2-1-8, 212232
2-1-9, 208244
2-1-10, 212212
2-1-11, 232232
2-1-12, 212212
2-1-13, 228228
2-1-14, 212212
2-1-15, 208216
2-1-16, 208208
2-1-17, 208216
2-1-18, 212212
2-1-19, 216216
2-1-20, 216216
Pop
3-1-1, 220224
3-1-2, 164192
3-1-3, 220240
3-1-4, 220260
3-1-5, 216256
3-1-6, 228240
3-1-7, 208240
3-1-8, 208208
3-1-9, 180180
3-1-10, 164220
Pop
4-1-1, 224236
4-1-2, 208228
4-1-3, 212212
4-1-4, 216232
4-1-5, 236264
4-1-6, 164212
4-1-7, 208208
4-1-8, 208228
4-1-9, 208208
4-1-10, 200224
4-1-11, 196196
4-1-12, 256256
4-1-13, 216216
4-1-14, 224244
4-1-15, 240240
4-1-16, 212224
4-1-17, 216232
4-1-18, 224224
4-1-19, 240256
4-1-20, 208224
Pop
5-1-1, 212236
5-1-2, 224236
5-1-3, 220232
5-1-4, 232244
5-1-5, 236236

5-1-6, 216238
5-1-7, 216228
5-1-8, 232240
5-1-9, 200208
5-1-10, 220236
5-1-11, 228228
5-1-12, 224236
5-1-13, 204212
5-1-14, 228240
5-1-15, 236236
5-1-16, 224236
5-1-17, 224232
5-1-18, 212240
5-1-19, 204224
5-1-20, 204212
5-1-21, 220232
5-1-22, 224236
5-1-23, 208228
5-1-24, 232232
5-1-25, 212228
5-1-26, 220224
Pop
6-1-1, 232244
6-1-2, 218240
6-1-3, 236244
6-1-4, 204224
6-1-5, 228232
6-1-6, 232232
6-1-7, 216240
6-1-8, 204220
6-1-9, 236236
6-1-10, 228256
6-1-11, 228228
6-1-12, 228228
6-1-13, 208244
6-1-14, 228228
6-1-15, 216244
6-1-16, 208236
6-1-17, 232232
6-1-18, 232232
6-1-19, 228236
6-1-20, 208228
6-1-21, 228236
6-1-23, 236252
6-1-24, 228232
Pop
2-2-1, 196204
2-2-2, 204204
2-2-3, 196204
2-2-4, 196196
2-2-5, 196204
2-2-6, 148196
2-2-7, 196196
2-2-9, 148196
2-2-10, 148196
Pop
3-2-1, 200200
3-2-2, 208240
3-2-3, 164212
3-2-4, 200240
3-2-5, 164212
3-2-6, 212236
3-2-7, 224240
3-2-8, 236236
3-2-9, 212240
3-2-10, 224224
3-2-11, 154200
3-2-12, 212228
3-2-13, 212232
3-2-14, 124200
3-2-15, 208220
3-2-16, 200216
3-2-17, 200216
3-2-18, 224236
3-2-19, 212216
3-2-20, 236236
3-2-21, 208228
3-2-22, 180236
3-2-23, 196224
3-2-24, 216228
3-2-25, 224236
Pop
4-2-1, 232256
4-2-2, 220220
4-2-3, 132208
4-2-4, 216216
4-2-5, 212232
4-2-6, 208224
4-2-7, 224224
4-2-8, 208208
4-2-9, 212232
4-2-10, 196196
4-2-11, 196220
4-2-12, 132208
4-2-13, 196228
4-2-14, 228228
4-2-15, 208224
4-2-16, 208208
4-2-17, 208216
4-2-18, 208208
4-2-19, 208208
Pop
5-2-1, 204212
5-2-2, 180188
5-2-3, 180188
5-2-5, 180188
5-2-6, 180200
5-2-7, 180200

5-2-8, 204220
5-2-9, 204212
5-2-10, 180188
5-2-11, 180208
5-2-12, 180188
5-2-13, 204212
5-2-14, 204204
5-2-15, 204224
5-2-16, 200200
5-2-17, 216216
5-2-18, 216220
5-2-19, 236236

Microsat on Etheostoma eca44EPA

Pop
1-1-1, 132140
1-1-2, 152156
1-1-3, 132140
1-1-4, 132144
1-1-5, 140148
1-1-6, 136140
1-1-7, 140148
1-1-8, 140148
1-1-9, 136144
1-1-10, 140148
1-1-11, 148152
1-1-12, 144152
1-1-13, 136144
1-1-14, 140144
1-1-15, 136144
1-1-16, 124124
1-1-17, 124132
1-1-18, 124128
1-1-19, 148148
1-1-20, 124140
1-1-21, 124132
1-1-22, 140144
1-1-23, 148148
1-1-24, 124124
1-1-25, 136148
Pop
2-1-1, 152160
2-1-2, 136136
2-1-3, 140148
2-1-4, 144144
2-1-5, 132140
2-1-6, 140148
2-1-7, 140156
2-1-8, 128136
2-1-9, 128136
2-1-10, 152160
2-1-11, 144148
2-1-12, 124128
2-1-13, 140140
2-1-14, 136148
2-1-15, 124136
2-1-16, 128128
2-1-17, 128128
2-1-18, 124148
2-1-19, 128128
2-1-20, 128136
Pop
3-1-1, 116122
3-1-2, 168168
3-1-3, 136136
3-1-4, 122130
3-1-5, 122130
3-1-6, 136144
3-1-7, 122130
3-1-8, 122130
3-1-9, 148148
3-1-10, 144144
Pop
4-1-1, 124140
4-1-2, 124132
4-1-3, 136144
4-1-4, 148152
4-1-5, 132140
4-1-6, 148168
4-1-7, 128136
4-1-8, 136144
4-1-9, 136144
4-1-10, 128136
4-1-11, 136144
4-1-12, 124156
4-1-13, 120168
4-1-14, 136144
4-1-15, 124132
4-1-16, 140148
4-1-17, 140148
4-1-18, 144148
4-1-19, 140148
4-1-20, 116128
Pop
5-1-1, 140164
5-1-2, 140148
5-1-3, 124148
5-1-4, 128140
5-1-5, 140140
5-1-6, 140148
5-1-7, 140156
5-1-8, 140140
5-1-9, 144156
5-1-10, 140140
5-1-11, 124156
5-1-12, 148148
5-1-13, 136160
5-1-14, 136136

5-1-15, 132144
5-1-16, 136156
5-1-17, 140140
5-1-18, 136156
5-1-19, 144156
5-1-20, 152160
5-1-21, 140164
5-1-22, 140156
5-1-23, 136144
5-1-24, 124144
5-1-25, 144152
5-1-26, 132144
Pop
6-1-1, 128152
6-1-2, 160168
6-1-3, 160168
6-1-4, 128136
6-1-5, 160168
6-1-6, 136144
6-1-7, 140140
6-1-8, 152162
6-1-9, 136136
6-1-10, 152162
6-1-11, 116116
6-1-12, 140148
6-1-13, 136180
6-1-14, 148148
6-1-15, 136148
6-1-16, 140148
6-1-17, 136140
6-1-18, 140148
6-1-19, 136152
6-1-20, 140144
6-1-21, 124144
6-1-23, 148152
6-1-24, 140160